ULTRASENSITIVE DNA APTASENSORS BASED ON EXPONENTIAL AMPLIFICATION REACTION

BY

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THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science in Chemistry
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2013

Urbana, Illinois

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ABSTRACT

This is a comprehensive project for the development of a general method to enhance the sensitivity of DNA aptasensors by coupling them with an isothermal DNA amplification technique known as exponential amplification reaction (EXPAR). The method involves two steps: (1) first, the target-induced structure-switching of DNA aptamers causes the release of trigger oligonucleotides from the aptamers immobilized on a surface; (2) then, the released trigger oligonucleotides are amplified by the EXPAR technique for ultrasensitive detection. It is shown that the detection limit of a cocaine aptasensor is improved from 5 micromolar in assays without amplification to 0.8-1micromolar when EXPAR is applied, providing a 5-fold enhancement. The flexibility in designing the sequences of the trigger oligonucleotides enables the method generally applicable to other aptasensor systems such as thrombin aptamers. By EXPAR, the method has also achieved 10-fold enhancement in sensitivity from 4nM to 400 pM for thrombin detection, in comparison with similar assays without EXPAR.

To my beautiful baby girl Yhalitzie Thaylee Perez-Brown

ACKNOWLEDGMENTS

My time spent at the University of Illinois at Urbana-Champaign was one of the most meaningful periods in my life. First of all I would like to thank and acknowledge the almighty God. His influence and teachings have help guide me through life, helped me become the man I am today and put me in the position where I am now to succeed and help make everyone involved with my life proud of what I am doing. Many thanks and blessings go out to my advisor, Dr. Yi Lu, whose patience and guidance throughout my time there at UIUC were admirable and of the utmost generosity. His ability to see what motivating factors influenced me most and to exhibit them so gracefully were what pushed me along through my program and allowed for me and my talents to blossom. I also need to thank and acknowledge all the Lu lab members especially those in the Beckman Institute including Longhua, Hang and Juanjuan who will be my friends for life. Mainly from the Lu Lab I would like to thank Yu Xiang who helped guide me through all my experiments and my entire experience in the lab, and listened to my off the wall craziness and silly suggestions. Without his help I would not have been able to succeed in grad school. I would also like to thank everyone from the chemistry graduate office especially Krista Smith who has also showed vital patience throughout my graduate school experience and helped me transition from graduate school to the real world. I would like to especially thank my wife Krystle Thaylee Perez who has endured the entire process I have been through since before Day 1 of grad school and all throughout and continuously offered her love and support. Finally, I would like to thank my friends Mathew Ward and Nicholas Santiago and family who never gave up on me and constantly checked up on my wellbeing while I was deep down in the trenches of my graduate program.

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CHAPTER 1

INTRODUCTION

A sensor is a device or molecule that responds to a chemical or physical stimulus, and converts that response to a measureable signal. To ensure in the health, safety and comfort of our society, sensors of all types have been critical and widely used for medical diagnosis, food safety and environmental monitoring. [1] With respect to these applications, various analytes throughout environment and biology are targeted and evaluated. Among many, these analytes include metal ions, toxins, viruses and small molecules. [1-5]

Some examples of the current technologies used to measure these analytes include High pressure liquid chromatography (HPLC) and Gas chromatography-Mass spectrometry (GC-MS).[1] These methods are used to separate different chemical compounds in a material in order to identify and quantify the substances within the material of interest, therefore providing a means of detection for specific targets. One advantage of using methods like these includes the high sensitivity they are capable of, sometimes down to parts per billion or less of a specific target. Also, they provide a standard for industries and have the ability to detect different targets simultaneously. However, the usage of these technologies also gives some disadvantages that are not desirable in some cases. One disadvantage is the requirement of highly sophisticating equipment needed to provide such high sensitivities. In addition, in these methods there is also a need for timely and costly sample pretreatment and professional personnel to carry out measurement and data analysis. These disadvantages make it impossible to provide the public with a portable and easy-to-use method to monitor the targets of interest. On the other hand, due to the growing need for real-time, on-site and *in situ*

detection of many targets, the development of a general method for the sensitive and selective recognition of a broad range of targets has also become of great importance for scientist in all fields, to avoid the complicated trial-and-error to develop different methods for different targets. [5]

In order to develop a general method for the ultrasensitive detection of a target of interest, a high quality sensor must be incorporated and utilized within the design of the overall method. A high quality sensor that can be converted into an efficient tool for detection will have many characteristics that must be accounted for. This sensor will be simple to handle and have a rapid analysis, being able to provide the user with a result very quickly and easily. It will be unsusceptible to its environment enabling its usage in a variety of locations and situations, portable to allow for detection on the go, and be highly sensitive and selective to its target. Also, a high quality sensor will have a tunable dynamic range to provide the ability to detect targets with a wide range of concentrations and most importantly be cost-effective. Most technologies used today integrate sensors which carry some of these features but not all of them together. So there is a need for a general method which uses a high quality sensor that combines all of these features into one and provides us with rapid, ultrasensitive and real-time detection and quantification for a broad range of targets.

CHAPTER 2

FUNCTIONAL DNA AND PERFORMANCE ENHANCING

One material that has been shown to not only be used as a sensor but also exhibits all of the qualities necessary to be considered a high quality sensor is a specific type of Deoxyribonucleic acid (DNA), known as Functional nucleic acids or also known as Functional DNA. [4-10] Since the discovery of functional nucleic acids in the early 90's, many have realized their potential as major components in the realm of biosensing. [4-6, 11] With this potential, DNA has launched itself out of its traditional spotlight of genetic information carrier, and into its newly pronounced operative role for environmental, pharmaceutical and biochemical applications. [4-6, 11] Functional DNA are a group of oligonucleotides consisting of aptamers, DNA enzymes (or known as DNAzymes or deoxyribozymes) and aptazymes with functions beyond the conventional roles of DNA as a genetic information carrier. [4-6, 11, 12] Using a combinatorial technique called in vitro selection or Systematic evolution of ligands by exponential enrichment (SELEX), nucleic acid sequences that exhibit catalytic behavior (DNAzymes), specifically bind to a target (aptamers), or both (aptazymes) have been developed. [4-6, 8-9] With in vitro selection, a desired target is incubated with a large random nucleic acid pool containing 10^{14}-10^{16} different sequences. Nucleic acid sequences that bind to the target are preserved while unbound sequences are removed using an affinity column immobilized with the target of choice. Next, the sequences that have bound to the target are eluted and amplified using polymerase chain reaction (PCR). [13] This cycle is repeated, and after many positive and negative selection rounds with increasingly stringent conditions, nucleic acid

sequences with a high binding affinity and specificity towards that desired target are obtained. [13] Through this procedure of in vitro selection, nucleic acids with the ability to change conformation and/or perform catalytic reactions at its core in the presence of specific inorganic, organic, biomolecules and even bacteria, cancel cells or viruses has been obtained and utilized for analyte detection (Simple demonstration along with table of targeting species exhibited in Figure 1 and Table 1). [11] Shown in Figure 2 is a depictive image of in vitro selection and examples of the end results of the active DNA with the corresponding molecule or target that is selective for that nucleic acid.

Functional DNA provides the platform for a high quality sensing device that can be incorporated into a general method for the detection of a target of choice. The basic characteristics of DNA are what make it attractive as a sensing device and allow for us to take advantage of in order to generate the high quality sensor. [6] The predictable Watson-Crick base pairing interactions give flexibility to the design of the overall sensor. [6] The low cost synthesis of DNA with a range of chemical modifications that can be used for the attachment of and utilization of assisting materials offers the means for a cost-effective detection method. Also, the high stability of DNA through hydrogen bonding and the fact that it can be denatured and renatured readily without losing its characteristics and ability to perform its designated duties has made DNA a highly capable material to integrate into a sensing technology. [11] Being that there is an established procedure for the synthesis of functional DNA that will be active towards a specific target of choice, the possibilities of targets to employ functional DNA for detection purposes are almost endless.

Functional DNA performing target recognition consists of one part of the sensing mechanism.[4-9] The second, signal transduction, can be easily achieved through the incorporation of fluorophores, quenchers and other signal transducing materials through various well known bioconjugation techniques. Because of the unique characteristics of materials such as the optical properties of Au nanoparticles (NP), the fluorescent emission of fluorophores and the reduction of fluorescent signal by quenchers, the target recognition process performed by functional DNA can be quantified and analyzed to determine the amount of a specific target is present in a given environment. [11] With the functional DNA responding to the physical stimuli of a specific target being recognized, and the signal transducing materials turning that response into a measurable quantity, functional DNA can be employed as sensors with tunable dynamic ranges for small analyte targets. With functional DNA based sensors, binding events are transduced into signals proportional to the concentration of analyte present in the system. These signals can thus be analyzed by the fluorescence, color, electric and magnetic properties that are associated with their specific transducing materials. [11] To give a complete picture, by combining functional DNA with certain materials that have very distinct properties, a sensing device that is not only selective and non-interfering with its target, but is also one that is cost efficient and readily available can be engineered. Functional DNA provides the platform for further studies to increase the number of target choices and overall capabilities of these types of sensors.

Many different detection methods have been developed based on target recognition in functional DNA and the corresponding response to physical stimuli. In this case, the stimulus is the target and the response is the catalytic behavior of the enzymatic core in DNAzymes and/or

the structure switching conformational change of the ssDNA in DNA aptamers. [11] With the generation of these nucleic acids in vitro, they can be engineered to have high selectivity to fundamentally any preferred molecule. [11] Also, being that the manipulation of DNA has been well researched and documented, signal transducing elements can be easily incorporated into the DNA sequence therefore satisfying the second requirement of a sensor device. Ultimately, we can obtain many types of signals including fluorescent, color, electric and magnetic dependant on the choice of integrated materials. Ongoing research with functional DNA-based sensors has resulted in many effective and reliable detection methods for a number of targets including environmental contaminants such as Pb^{2+} [8, 14], radioactive species such as uranyl (UO_2^{2+}) [15], and small organic molecules of interest such as cocaine [16-19] and adenosine. [20] Examples of two different functional DNA based sensors developed for the detection of Pb^{2+} using different signal reporters is shown in Figure 3. [8, 14] Despite the pronounced success of these types of sensing technologies, the increasingly extreme and stringent sensitivity requirements needed to satisfy personal, environmental, and governmental standards have limited the usage of functional DNA-based sensors in forensic and clinical applications. In general, though functional DNA provides a great platform for the responsive and discriminatory detection of a vast range of analytes, there is still a need for performance enhancement of these systems.

Functional DNA based sensors are a great start but do not quite provide us with a powerful enough tool for detection and quantification of targets of interest for forensic and clinical applications. With the current limits of detection (LOD) functional DNA based sensors offer for various molecules and species there is a need for sensor performance enhancement.

Being that functional DNA was discovered in the 90s, there have been ongoing and exhausting efforts on the improvement of these biosensors and their performance. [4-9, 11] One way in which performance has been progressed is through the coupling of already established procedures with these sensors. Due to the nature of functional DNA-based sensors, that being the inclusiveness and manipulation of nucleic acids, the amplification of DNA has become a valuable asset in the improvement of such systems. [21] In these systems, the amplification of DNA generates an increase in signal resulting in an enhancement of the overall performance of that system. Owing to the fact that isothermal DNA amplification is a more cost-effective, rapid and simple method than the more established thermocycling technique of polymerase chain reaction (PCR), it has become a prime candidate for the performance enhancement of functional DNA based sensors. [22-31] In isothermal DNA amplification the amount or concentration of DNA is amplified through means of a DNA polymerase at a constant high temperature. [21] Isothermal DNA amplification is highly sensitive and selective and does not require thermally stable DNA polymerases or sophisticated equipment. These properties make the coupling and compatibility of this amplification method with functional DNA-based sensors a more facile process. By coupling this technique with functional DNA based sensors, one is able to increase the amount of DNA present in the environment which will then increase the amount of signal being reported to the transducing materials, thus enhancing the performance of the sensor, decreasing the LOD and increasing the overall sensitivity of the sensing device. [32] This entire process of using functional DNA based sensors that can be generated to detect essentially any molecule of choice and coupling them with isothermal DNA amplification to enhance the LODs of that sensor provides a general methodology that in future projects can be

incorporated into a device for rapid, ultrasensitive, real-time, on-site and *in situ* detection of harmful and/or helpful targets of interest.

There have been many procedures established for isothermal DNA amplification. Some of those include Loop-mediated Isothermal Amplification (LAMP) in which a DNA polymerase and four turn back primers recognize six distinct DNA sequences [22, 23]; Rolling Circle Amplification (RCA) in which a circular template is replicated hundreds to thousands of times; [24] Strand Displacement Amplification (SDA) in which a restriction endonuclease is employed to 'nick' a single bond in a DNA backbone and subsequently cause the release of a short single DNA strand. [25] Each of these methods has its advantages, and some such as RCA have been used alongside aptamers for the ultrasensitive detection of targets such as ATP (Adenosine Triphosphate) [26], platelet-derived growth factor (PDGF) [26], and cocaine. [31] Though some of the LOD's using isothermal DNA amplification techniques have reach low numbers, the time at which the assay takes place is too long in some cases and the ability to create an exponential reaction which can provide yet lower LOD's is absent with these techniques. Therefore, the development of a rapid and general ultrasensitive detection method for a broad range of targets, using functional DNA-based sensors enhanced with isothermal DNA amplification is still a challenge that needs to be met.

One way in which this challenge can be met is through the coupling of isothermal Exponential Amplification Reaction (EXPAR) with established functional DNA based sensors. EXPAR is a molecular chain reaction wherein the products of one reaction catalyze further reactions that generate the same products. It is an isothermal DNA amplification method in

which short ssDNA 10-25 nucleotides long are multiplied 10^6 times in less than five minutes. [27-30] This is accomplished through the employment of a DNA polymerase, a nicking enzyme, and a DNA template of simple design. When a trigger oligonucleotide (DNA strand to be amplified) is introduced into the system, a transient duplex is formed with the complementary DNA template. This transient duplex serves as the primer and activates the DNA polymerase. Once the recognition sequence is polymerized, the restriction endonuclease (nicking enzyme) is activated and hydrolyzes only one strand of the DNA duplex. [33] When this happens, the half of the DNA duplex is now below its critical length and the newly formed ssDNA releases from the transient duplex to become the reactant for further replication, thus creating an exponential effect (EXPAR shown in Figure 4). This effect can be quantified based on the time at which the amplification takes place and intercalating DNA dyes. The higher the starting concentration of DNA the faster the timing of the amplification will take place. Being that the DNA polymerase and nicking enzyme operate optimally at the same temperatures the reaction in entirely is performed at that temperature. The only requirements for the design of the EXPAR method is that the template must contain the recognition site which is contingent to the nicking enzyme of choice, 9 base pairs of cDNA on both sides of the recognition site and 3' blocking with either PO_4 groups or tethered Amines. These simple requirements enable easy coupling of the amplification system with functional DNA based sensing systems. By using aptamer structure switching mechanisms and solid supports for bioseparation, it is possible to couple the two procedures of target detection and isothermal DNA amplification, allowing for an ultrasensitive detection method that ultimately lowers the already established LOD for the functional DNA based sensor used for that target without any performance enhancers.

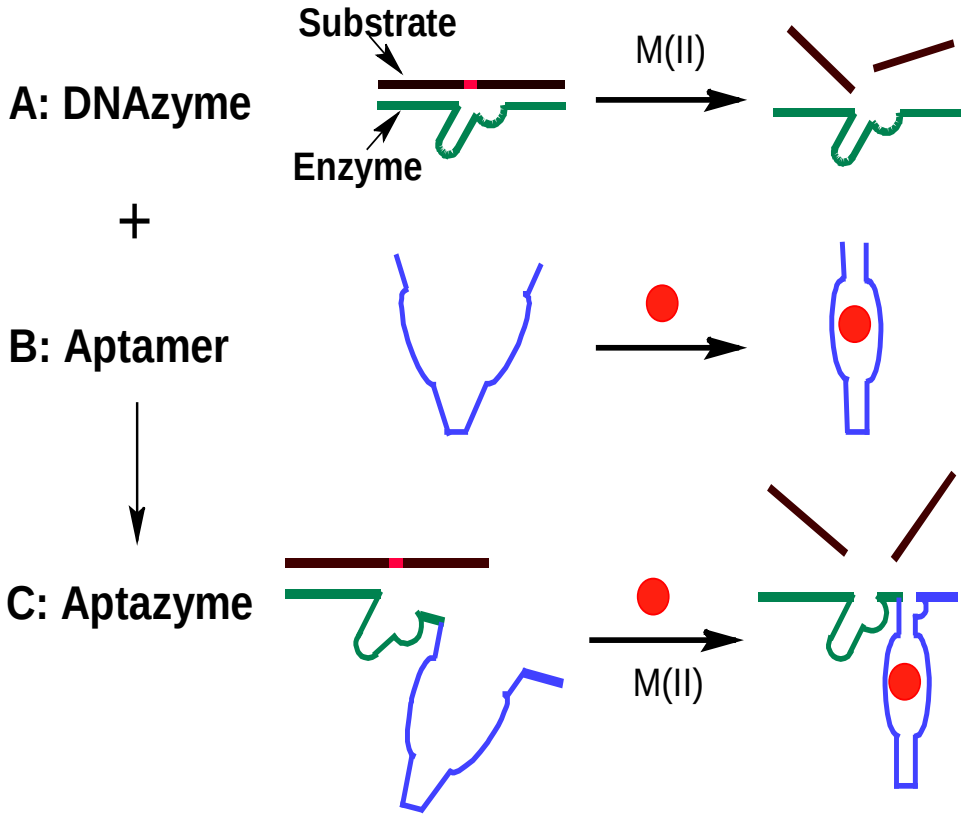


Figure 1. Functional nucleic acids as targeting agents

Examples of functional DNA performing A. catalytic reactions (DNAzyme), B. changing conformation (aptamer) or C. both in the presence of analyte targets (aptazyme)

Figure adapted from Lu Lab files and information obtained in:

Liu J, Cao Z, Lu Y, "Functional nucleic acid sensors", *Chem. Rev.,* **2009**, 109, 1948-1998

Contaminant type	Examples
Metal Ions	**Pb^{2+}, Cu^{2+}, Hg^{2+}, As^{5+}, Zn^{2+}**
Radionucleotides	**UO_2^{2+}**
Toxins	**Ricin, Abrin toxin, Microcystin**
Antibiotics	**Vasopressin, Streptomycin, Tetracycline, Viomycin, Chloramphenicol**
Endocrine disrupting compounds and hormones	**17β-estradiol, Thyroxine hormone**
Protein	**HA 1 proteins or H5N1 influenza virus, Thrombin**
Other small organic molecules	**Cocaine, cholic acid, (R)-thalidomide, Ethanolamine**
Cells and bacteria	**Anthrax spores, Campylobacter jejuni**

Table 1. Analytes targeted by functional DNAs

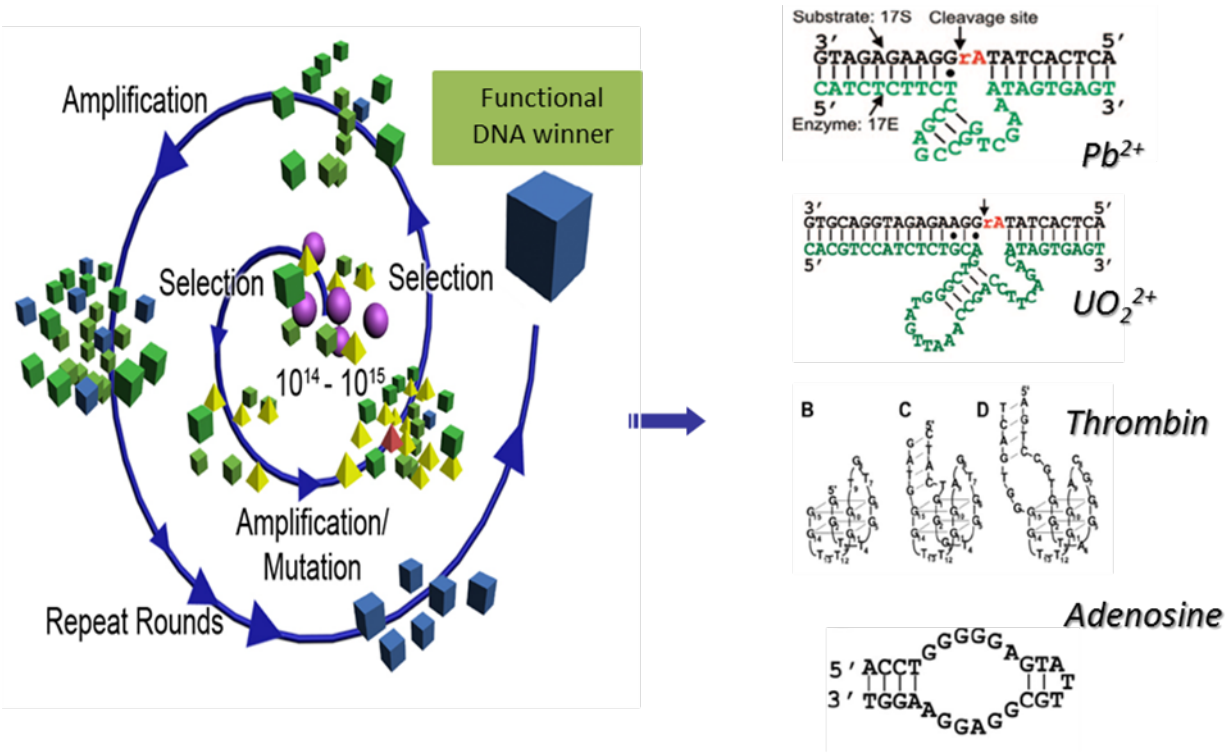


Figure 2. In vitro selection and examples of functional DNAs

Left: Schematic overview of in vitro selection process

Right: Examples of finished product of in vitro selection for DNAzymes (Pb^{2+}, UO_2^{2+}) and DNA aptamers for small molecule adenosine and protein thrombin

Figure adapted from:

Liu J, Cao Z, Lu Y, "Functional nucleic acid sensors", *Chem. Rev.,* **2009**, 109, 1948-1998

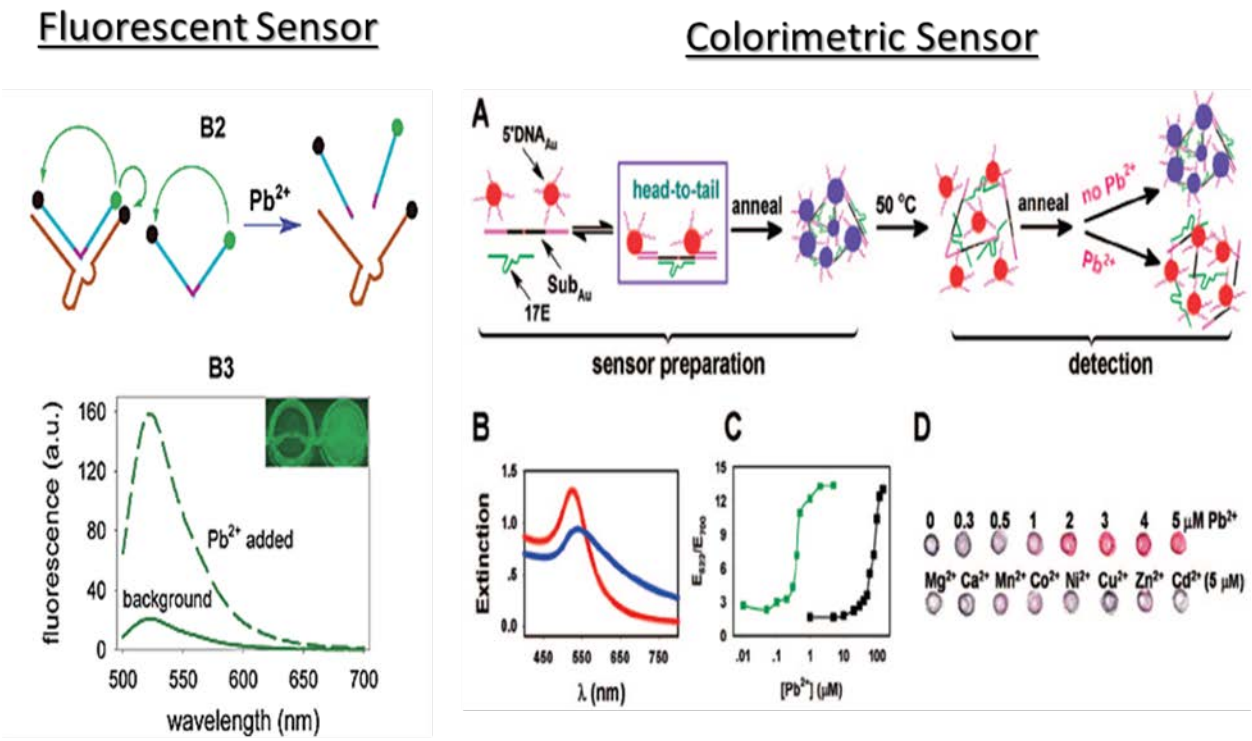


Figure 3. Functional DNA sensors for Pb^{2+}

Left: Fluorescent based DNAzyme sensor for Pb^{2+} ion and resulting graph

Right: Colorimetric based DNAzyme sensor for Pb^{2+} ion and resulting graphs

Figure adapted from:

Liu J, Cao Z, Lu Y, "Functional nucleic acid sensors", *Chem. Rev.,* **2009**, 109, 1948-1998

Liu J, Lu Y, "Improving Fluorescent DNAzyme Biosensors by Combining Inter- and Intramolecular Quenchers", *Anal. Chem.,* **2003**, 75, 6666-6672

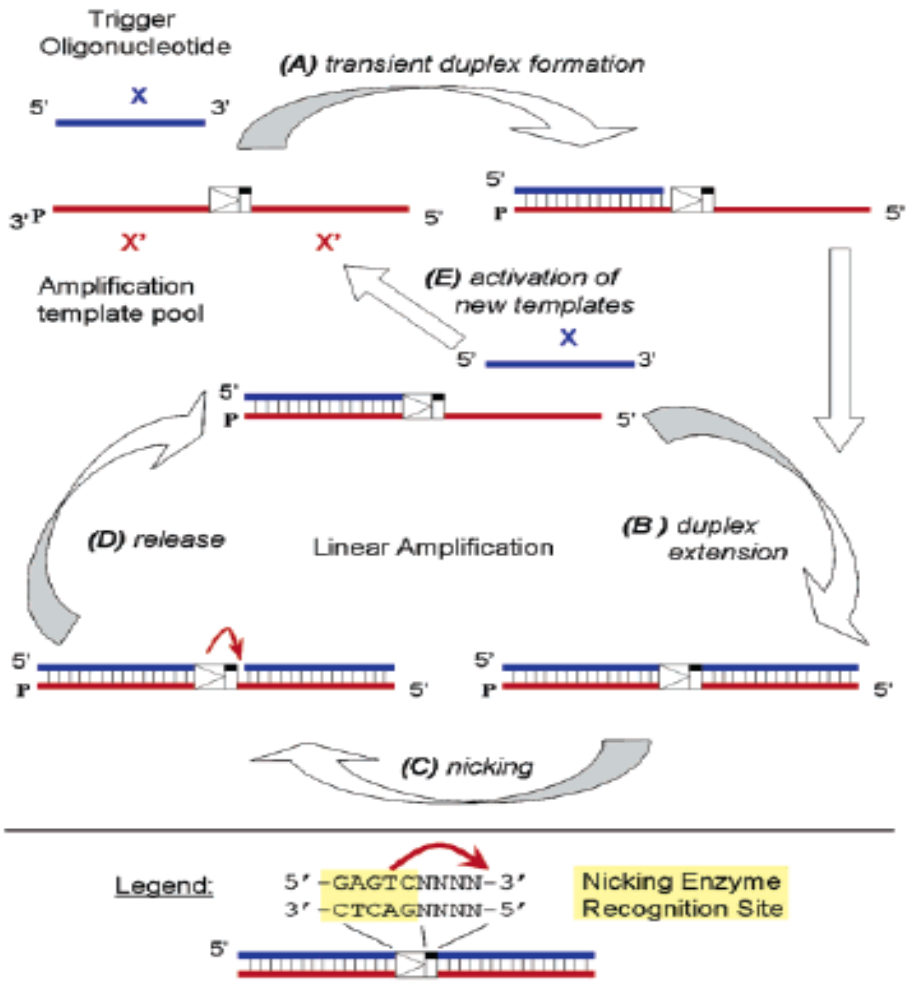


Figure 4. Exponential Amplification Reaction (EXPAR)

Schematic overview of Exponential Amplification reaction

Figure adapted from:

Tan E, Wong J, Nguyen D, Zhang Y, Erwin B, Van Ness LK, Baker SM, Galas DJ, Niemz A, "Isothermal DNA amplification coupled with DNA nanosphere-based colorimetric detection", *Anal. Chem.*, **2005**, 77, 7984-7992

CHAPTER 3

METHODOLOGY

Herein is the presentation of a general method for the enhancement of the LOD of the DNA Aptasensors for small molecule cocaine and the protein thrombin. The overall procedure for performance enhancement is achieved in three steps. For the detection of cocaine the first step (1) is sensor preparation which involves the bioconjugation of the functional DNA to a solid support. Due to the high binding affinity and selectivity of biotin-streptavidin interactions, a biotin labeled aptamer was conjugated to the solid surface of a streptavidin coated magnetic bead (MB). Multiple rounds of washing are performed to remove unbound DNA, followed by the hybridization of a complementary DNA strand (Trigger X) to the aptamer (Figure 5A). More washes are executed to assist in the reduction of background noise present from unhybridized Trigger X being present. The next step (2) commences with the introduction of the solutions containing different concentrations of the cocaine target into the system. The aptamer then undergoes a structure switching process due to its binding with the target. This leads to the release of Trigger X from the MB/aptamer complex and with magnetic separation, trigger X can be easily removed from the system for subsequent reactions. The final step (3) is the insertion of Trigger X into the EXPAR master mixes for simultaneous amplification and quantification of Trigger X. With this procedure, fluorescence spectroscopy is used for the analysis of Trigger X production by an intercalating dye SYBR Green II, which in turn allows us to assign a LOD based on the time at which Trigger X is amplified. The LOD using this procedure is justified by the theory that the higher the concentration of the target present in solution, the higher the

concentration of Trigger X that will be released from the complex for insertion into the EXPAR system and thus the faster the time of amplification will occur.

There are two keys to the success of this procedure. First, by using magnetic beads we allow for a fast and facile way to separate Trigger X from the detection system and combine it with the amplification system. Second is the design and operation of Trigger X. Being that the EXPAR template will amplify a specific single nucleic acid we can use that feature to create a Trigger X sequence that will couple the two systems and enable optimization of both systems separately and simultaneously. The Trigger X for this procedure is a single DNA strand specifically designed to be compatible in both the detection system and the amplification system. It contains a short 10-14 nucleotide DNA sequence complementary to the cocaine aptamer (cDNA) and a 10 nucleotide DNA sequence to be used in EXPAR (AmpDNA) separated by a poly$(A)_6$ linker. The design of Trigger X (Figure 5B) provides flexibility in the design of the detection scheme and also avoids interrupting the thermodynamic stability of the AmpDNA to the template during the formation and extension of the transient duplex in the EXPAR scheme. This allows for generalization of the detection system to perform optimization of the system and for utilizing other aptamers for the detection of different targets of interest.

The sensor preparation consisted of determining how many base pairs on the Trigger X species would allow for optimum background reduction and release of Trigger X. It contained experiments utilizing the streptavidin-coated MBs, biotin labeled cocaine aptamers and FAM labeled Trigger X DNA sequences with varying lengths and positions of complementary nucleotides (Figure 6). This experiment was done for target recognition of the conjugated

functional DNA and Trigger X optimization and is successful because of the quenching effect magnetic beads have on a fluorophore (FAM in our case) and because of the Trigger X release from the MB/Aptamer complex due to structure switching. These phenomena's along with the predictability provided through Watson-Crick base pairing allow for our system to be evaluated accordingly. Once the cocaine target is presented to the MB/aptamer complex and a conformational change occurs, the FAM labeled Trigger X will exhibit an increase in its fluorescence intensity. This increase is then quantified based on the initial fluorescence intensity provided by the overall complex before the addition of the target. The entire preparation of the sensor along with the detection of cocaine is performed in one buffer termed Detection Buffer (50 mM Tris/ 150 mM NaCL/ 0.05% Tween 20/ pH = 7.15). To start the sensor preparation, MBs are washed with the detection buffer twice. Then the streptavidin coated MBs are incubated with biotin labeled DNA aptamer for 30 minutes. By using magnetic separation the MB/Aptamer complex is washed two more times with the detection buffer, and incubated with 2µM Trigger X for 1 hour. Next, the complete sensor complex being the MB/Aptamer/Trigger X is washed three more times to remove any excess Trigger X. Finally, the addition of cocaine containing detection buffer enables a fluorescent readout dependant on the amount of target present. At this point, the initial LOD is determined via 3ϭ plus the average of blank to ensure comparison with similiar methods and targets in current literature and to provide a base line for the assessment of performance enhancement.

The ability to amplify the newly designed Trigger X via EXPAR was evaluated to ensure the designs functionality in the EXPAR system. This evaluation was performed in the designated master mixes according to the enzymes used (Bst and Nt.BstNBI) and previous

literature. [27-30] Each master mix, mix A [1X NEBuffer 3 (50 mM Tris-HCl/ 100 mM NaCL/ 5 mM $MgCL_2$/ 1 mM Dithiothreitol/ pH = 7.9), 2.4 mM EGTA, 500uM dNTPs, 200 nM X'-X' DNA Template, and the mixed in Trigger X] and mix B [1X DNA Polymerase Buffer (20 mM Tris-HCl/ 10 mM $(NH_4)_2SO_4$/ 10 mM KCl/ 2 mM $MgSO_4$/ 0.1 % Triton X-100/ pH 8.8), 0.8 unit/mL Nt.BstNBI nicking enzyme, 0.16 unit/mL Bst DNA polymerase, 1X SYBR Green II dye] was prepared in an ice bucket directly before mixing. SYBR Green II dye is used as the signal tranducing material. To initiate the reaction, Trigger X is diluted into Mix A. Next, equal amounts of Mix A and Mix B are mixed together and immediately placed into the heating chamber where the samples are preheated for 75 seconds prior to analysis. Measurements are taken in 5 second intervals for a total of 5 minutes and the entire reaction takes place at 55 ^oC.

To complete the entire procedure for the ultrasensitive detection of cocaine, the sensor complex is prepared and the target is recognized same as described previously. After target recognition, Trigger X is removed from the MB/Aptamer complex by magnetic separation and diluted 25X into millipore H_2O and then 10X into Mix A. This dilution is done to provide minimum interference of any materials present from the detection system to the amplification system. Next, the Trigger X containing Mix A is combined with an equal volume of Mix B. Finally the system is heated to activate amplification, and analyzed. The total assay time for the detection of the target and amplification of Trigger X is less than 30 minutes. The LOD for the overall method of target recognition and amplification is calculated and compared to the LOD for the detection system alone. This LOD is calculated similarly to the LOD with the FAM labeled Trigger X, except since it is based on the point of infections (PoI) of the amplification of Trigger X, which decrease with increasing analyte concentration, it is defined as three times the

standard deviation of the blank minus the concentration of analyte required to give a signal equal to the background (blank) sample.

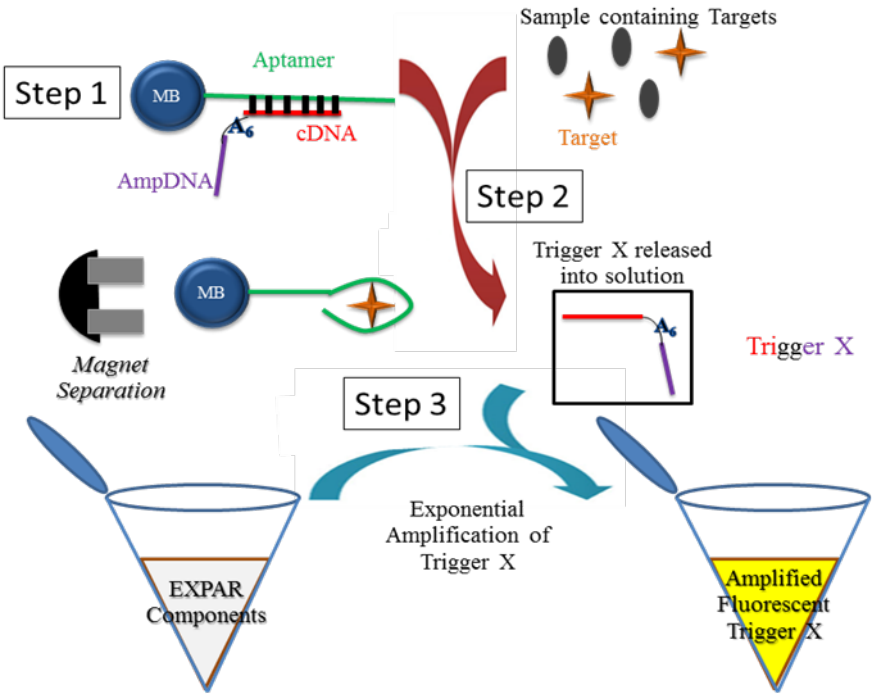
In order to establish the generalization of this overall method the thrombin aptamers were chosen to demonstrate the 'sandwich' based detection of thrombin with a slightly different procedural design and minimum modification of the Trigger X used. Instead of using MBs, a neutravidin coated 96 well plate was used as the solid support and the biotin labeled aptamer was replaced with the desthiobiotin labeled thrombin aptamer (15mer). This allowed for the release of the thrombin sensing complex from the plate which is then inserted into the EXPAR system for amplification. The Trigger X design was similar to the cocaine procedure in that it contained a 10 nucleotide sequence complementary to the same DNA template and a poly$(A)_6$ linker. The difference in this procedure was the use of the 29mer thrombin aptamer linked to the AmpDNA instead of the cDNA to the cocaine aptamer. In this procedure, the desthiobiotin labeled 15mer thrombin aptamer would bind to the neutravidin coated plates through the strong avidin-biotin interaction. After rounds of washing to remove unbound sequences, the complex is then exposed to solutions containing different concentrations of thrombin. The higher the concentration of thrombin, the more target loading will take place onto the complex. Next, the Trigger X containing the 29mer thrombin aptamer is added to the solution and after structure switching and conformational changes of the 29mer thrombin aptamer a sandwich complex of the thrombin between the two aptamers is present along with the AmpDNA for the following EXPAR reaction. This complex is then immersed with a biotin containing solution releasing it from the plates. Finally this solution containing the entire complex including the AmpDNA is inserted into the EXPAR system for amplification and

quantification of the concentration of target present. The evaluation of target recognition is accomplished similarly to the cocaine procedure with a FAM labeled Trigger X species. The presence of thrombin increases the load amount of FAM labeled Trigger X and subsequent releasing of the sensing complex results in a spike in fluorescence intensity of the solution. The ratio of initial fluorescence of the solution prior to releasing to final fluorescence intensity of the solution after releasing provides a measure of target recognition. The amplification of the Trigger X being used in this procedure is evaluated exactly the same way as in the cocaine procedure.

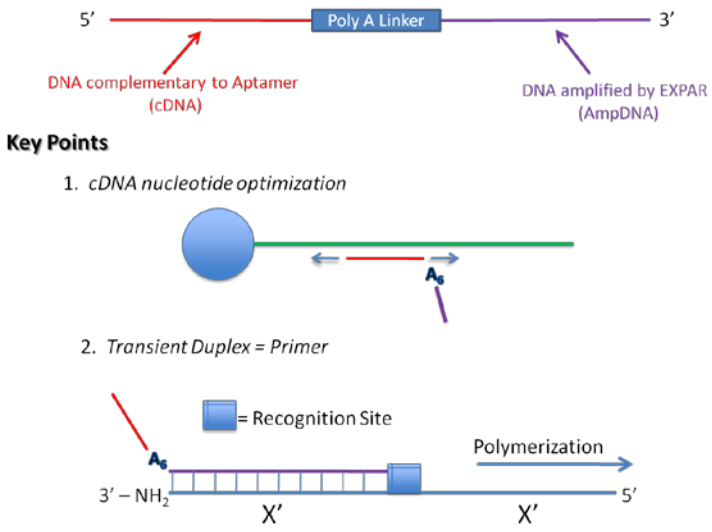
The DNA used for both procedures was purchased from IDT DNA Technologies, while the components of the master mixes including buffers, Bst DNA polymerase and the Nt.BstNBI Nicking Enzyme were all purchased from New England Biolabs in Ipswich, MA. The magnetic beads were purchased from Bangs Laboratories, the neutravidin coated plates were purchased from Thermo Scientific and all other reagents were from Sigma. Experiments were done on a FluoroMax-P fluorimeter (HORIBA Jobin Yvon Inc., Edison, NJ) fit with a heating chamber and an Analyst HT at the High Throughput Screening Facility of the School of Chemical Sciences in Noyes Laboratory at the University of Illinois at Urbana-Champaign.

CHAPTER 3 FIGURES AND TABLES

Figure 5. Overall procedure for cocaine detection and Trigger X design

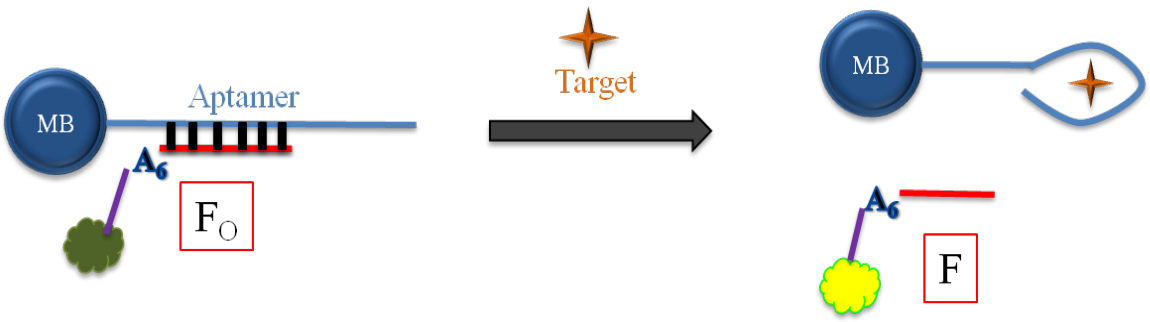


A. Overall step-wise procedure for target detection and amplification of Trigger X



B. Trigger X design and key points to Trigger X model

Figure 6. Target recognition and Trigger X optimization



X12 3'-C CTA CGA CTG **AAA AAA** TAG AGC CCT CTG -5'

X13C 3'-C CTA CGA CTG **AAA AAA** TAG AGC CCT CTG **T** -5'

X13T 3'-C CTA CGA CTG **AAA AAA** **T** TAG AGC CCT CTG -5'

X14 3'-C CTA CGA CTG **AAA AAA** **T** TAG AGC CCT CTG **T** -5'

Schematic of aptamer target recognition and subsequent structure switching and release of FAM-labeled Trigger X

Four different DNA sequences (X12, X13C, X13T, X14) for Trigger X optimization containing varying lengths and locations of extra nucleotides (enlarged and in Bold) and poly $(A)_6$ linker (underline)

CHAPTER 4

RESULTS AND DISCUSSION

For the cocaine detection system to be successful it is important to find the optimal conditions for proper loading of the aptamer onto the solid surface and for efficient hybridization of the cDNA with the aptamer. With incorrect or inadequate preparation of the device, the activity of the aptamer can be lost. In accordance with this, different conditions were tested for the hybridization of cDNA and target recognition of cocaine. To evaluate our sensor's performance when using only the detection probe without EXPAR, we used a FAM labeled Trigger X, which upon release from the MB/Aptamer complex produces a fluorescence signal in solution. Figure 7 shows the results of using the four differently sequenced cDNAs based on varying lengths and positions of nucleotides shown in Figure 6. Using four different sequences of Trigger X makes it possible to probe the balance between the low background and high sensitivity of our device, and thus improving our sensor performance. All the Trigger X sequences shown in Figure 7 show little and similar background intensity (0 μM cocaine). However, Trigger X sequences X13C and X14 contain an extra base pair on the cocaine aptamer sequence and show a decrease in sensor performance. Trigger X sequences X12 and X13T contain none or 1 base pair on the PolyA linker and show an increase in sensor performance. Therefore, adding complementary DNA base pairing to the cocaine aptamer sequence decreased sensor performance and in order to preserve and/or enhance performance, additions to the cDNA must be made to the 3' side of the DNA sequence. To provide a performance evaluation of the detection system prior to DNA amplification, Figure 8 shows the

graphs of the increase in fluorescence intensity as well as the background to signal ratio of the system using X13T as our Trigger X with the addition of increasing cocaine concentration. As the concentration of cocaine in the micro molar range is increased, the fluorescent intensity of the system is increased as well. These results show that optimization of our detection system in regards to the balance between a low background noise and a high sensitivity is achieved and a dynamic range for the sensor is obtained**.** Overall, the performance of our detection system is comparable to similar technologies that use fluorescence based analyses of cocaine with a LOD = ~5µM. The limit of detection is defined by the concentration of analyte required to give a signal equal to the background (blank) sample plus three times the standard deviation of the blank. By using a calibration curve the concentration LOD is calculated from the best fit line equation.

The ability to amplify Trigger X with conventional EXPAR conditions and also with the EXPAR conditions needed for our system was performed. In our system, Trigger X is coming from the detection buffer and must be diluted into our master mixes. This is a concern because of the sensitivity of the enzymes used in EXPAR. Figure 9 represents the graph of a typical EXPAR experiment in which the time at which amplification occurs is dependent on the concentration of Trigger X13T present in the system. As the concentration of Trigger X13T is increased from 10pM to 10nM, the time at which that sample is amplified increases proportionately. For high accuracy and resolution of the amplification, a plot of PoIs corresponding to maximum slope of the fluorescence curves is also shown in Figure 9. Through EXPAR we achieved amplification of AmpDNA only and Trigger X13T (cDNA + PolyA_6 + AmpDNA) down to pM levels. Due to the amount of Trigger X being released from our detection scheme,

pM amplifications of Trigger X is more than sufficient to amplify the DNA from our detection system. The amplification of Trigger X13T diluted in the buffer used for the detection steps is still attainable as shown in Figure 9.

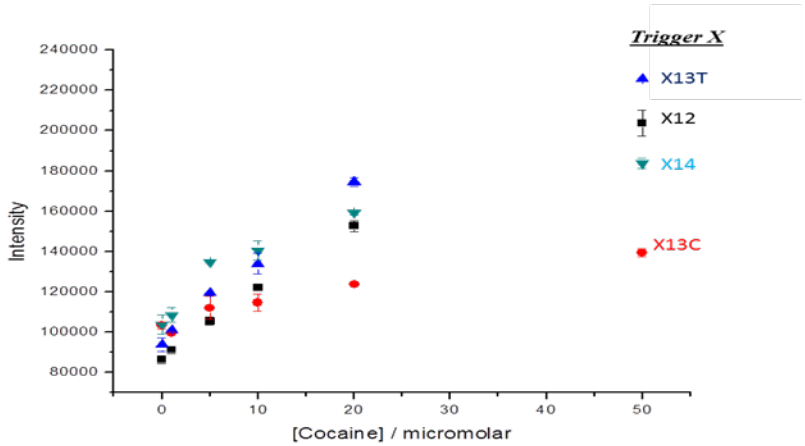
Performing the overall procedure simply consists of target recognition and Trigger X release, followed by magnetic separation and Trigger X introduction into master mixes. Through this process, we are able to successfully couple a detection system with an amplification system. Shown in Figure 10, as the concentration of cocaine is increased from 0-500µM, the time at which the sample is amplified increases. To clarify, the blank sample on this graph represents an EXPAR performed without the addition of any solution from the detection system, while the 0µM is an EXPAR performed with the addition of the blank sample from the detection scheme (no cocaine added to the MB/Aptamer complex). Figure 10 also contains the corresponding PoI graph. The LOD for cocaine for the overall procedure described herein is calculated to be ~800nM-1µM giving a 5X enhancement in the LOD for this aptamer and target versus the method without using amplification. This LOD is calculated similarly to the LOD with the FAM labeled Trigger X, except since it is based on the PoIs of the amplification of Trigger X13T, which decrease with increasing analyte concentration, it is defined as three times the standard deviation of the blank minus the concentration of analyte required to give a signal equal to the background (blank) sample.

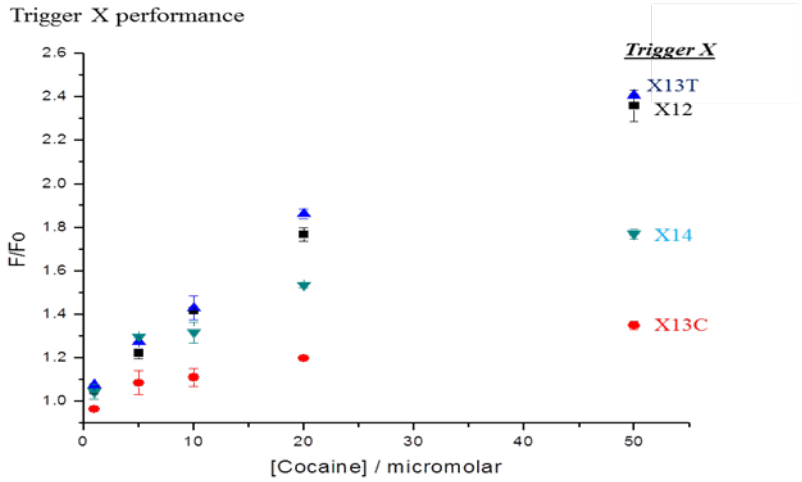
Using the procedure described for the thrombin detection, the target recognition was successful and the performance of the functional DNA based sensor yielded a LOD = 4nM, which is also comparable to current technologies used for these aptamers and target without

any amplification. The graphs shown in Figure 11 show the spike in fluorescence intensity and also an increase in the background to signal ratio with increasing concentrations of thrombin from 0-500nM and provides a dynamic range for this sensing device. The amplification of the Trigger X for the thrombin detection method [Thrombin Aptamer(29) + PolyA_6 + AmpDNA] was also successful as shown in Figure 12 by amplifying concentrations of the Trigger X down to the low pM range. The amplification of this Trigger X which contains 38 non-active nucleotides confirms that this type of Trigger X model can be utilized for enhancing other functional DNA based sensors and provides generalization within the procedural designs for other targets. The overall procedure with the target recognition of thrombin and the amplification of the DNA after being released from the plates was accomplished as shown in Figure 13. Using the PoI graph the LOD for this overall method is calculated to be 400pM which give a 10X enhancement over the initial detection system.

CHAPTER 4 FIGURES AND TABLES

Figure 7. Trigger X optimization.



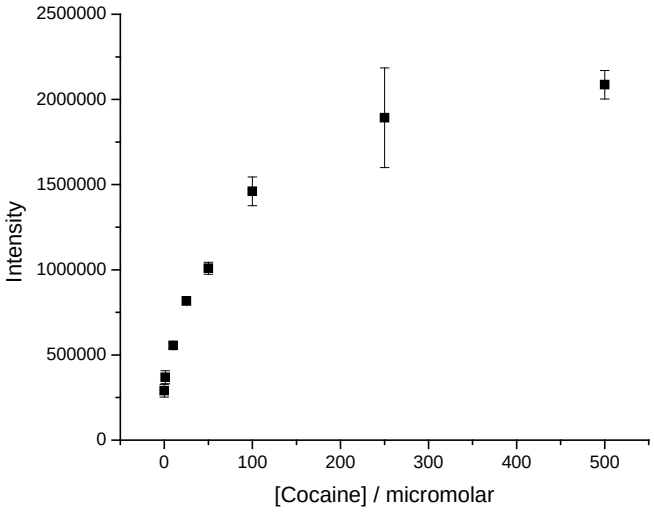


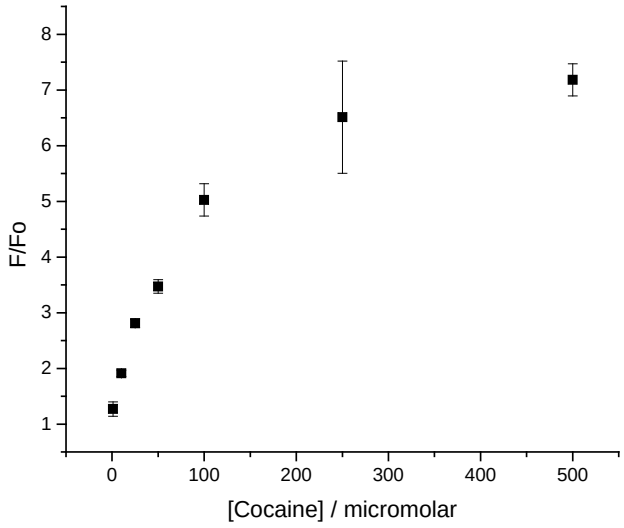
Top: Graph of fluorescence intensity increase of different FAM labeled Trigger X's with increasing [cocaine] = 0-50µM

Bottom: Graph of background to signal ratio of same Trigger X's with increasing [cocaine]

Experiment performed with 1mg/mL of Magnetic beads, 1.75µM biotin labeled cocaine aptamer, 2µM FAM-lebeled Trigger X and 30 minute incubation with buffer containing varying [cocaine]

Figure 8. Trigger X13T performance



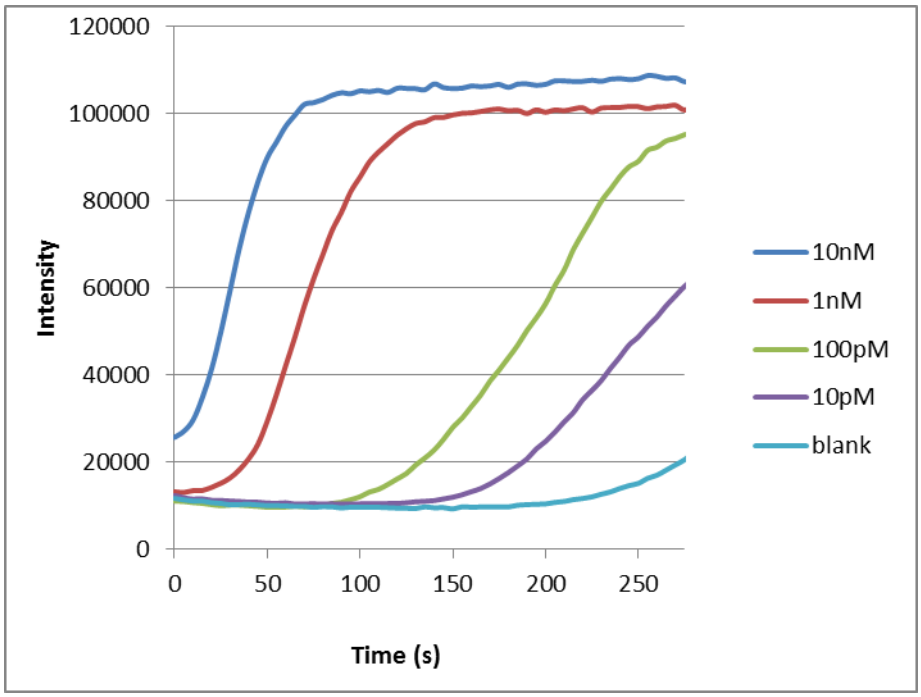


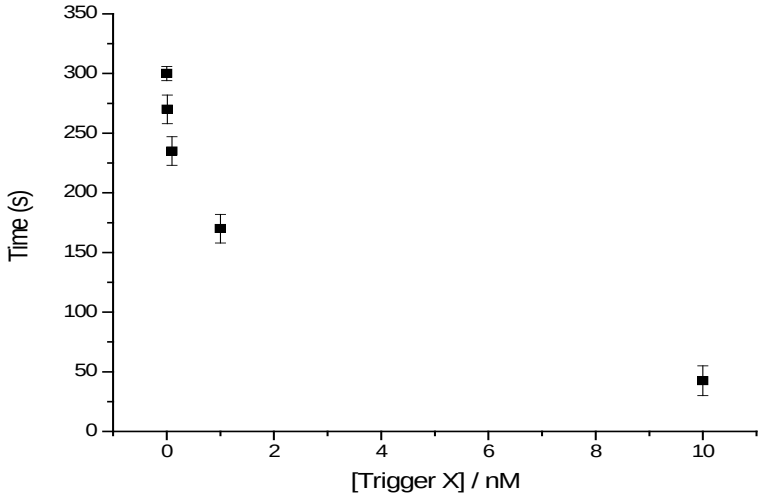
Top: Graph of fluorescence intensity increase of Trigger X (X13T) with increasing [cocaine] = 0-500µM

Bottom: Graph of background to signal ratio of same Trigger X with increasing [cocaine] = 0-500µM

Experiment performed with 1mg/mL of Magnetic beads, 1.75 µM biotin labeled cocaine aptamer, 2µM FAM-labeled Trigger X and 30 minute incubation with buffer containing varying [cocaine]

Figure 9. Trigger X13T amplification via EXPAR and corresponding PoI graph



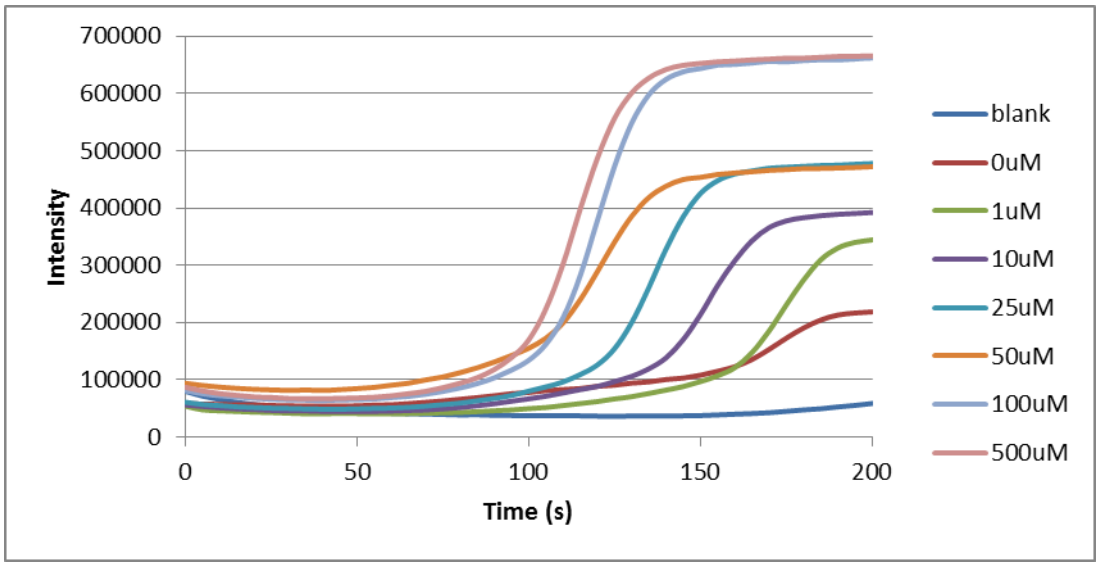


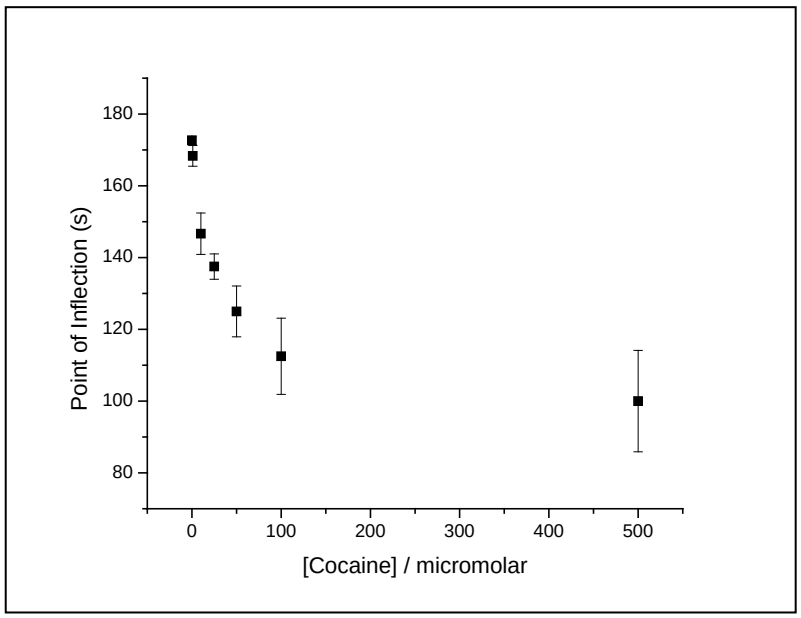
Top: Graph of fluorescence intensity vs. time for varying [Trigger X13T]

Bottom: Graph of Inflection points corresponding to maximum slope of the fluorescence curves from top graph

Experiment performed by combing equal amounts of master mixes A and B and diluting Trigger X13T with millipore water to obtain its desired concentration prior to its insertion into master mix A. Samples analyzed for 5 minutes at 55°C with fluorescence excitation of 490nm and emission 520nm

Figure 10. *Overall procedure:* EXPAR of Trigger X13T released from sensor complex



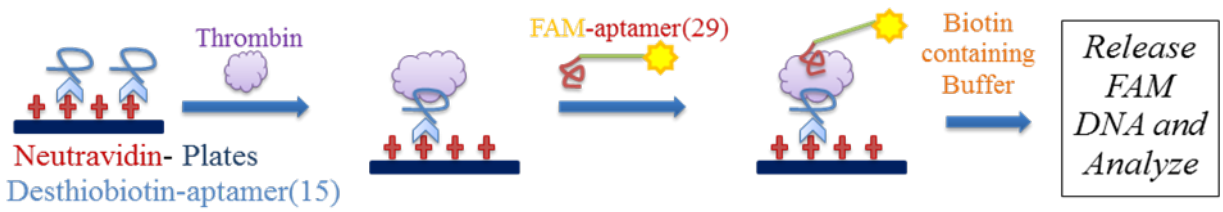


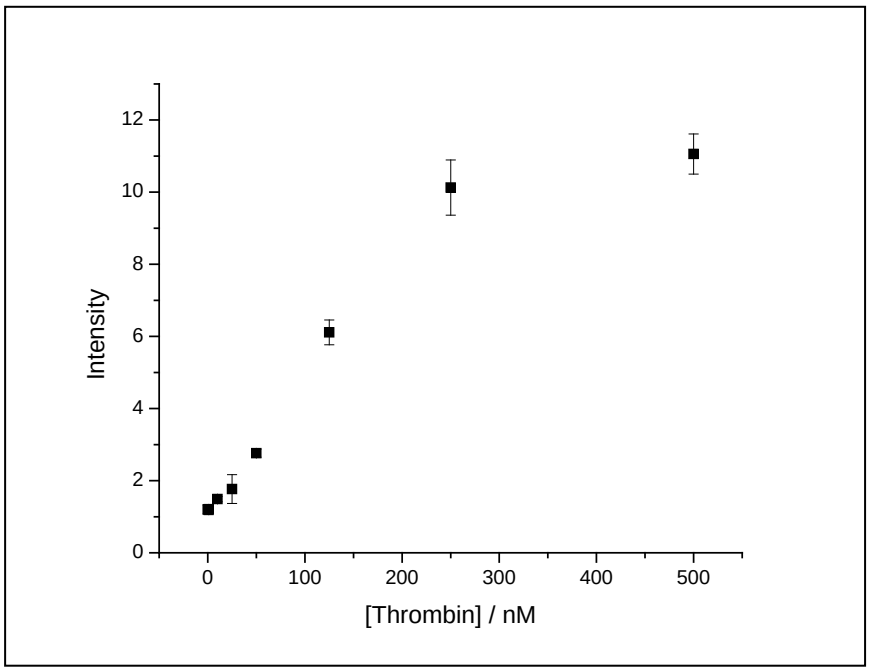
Top: Graph of fluorescence intensity vs. time for Trigger X13T obtained from target induced structure switching of cocaine aptamer containing varying [cocaine], 0-500µM

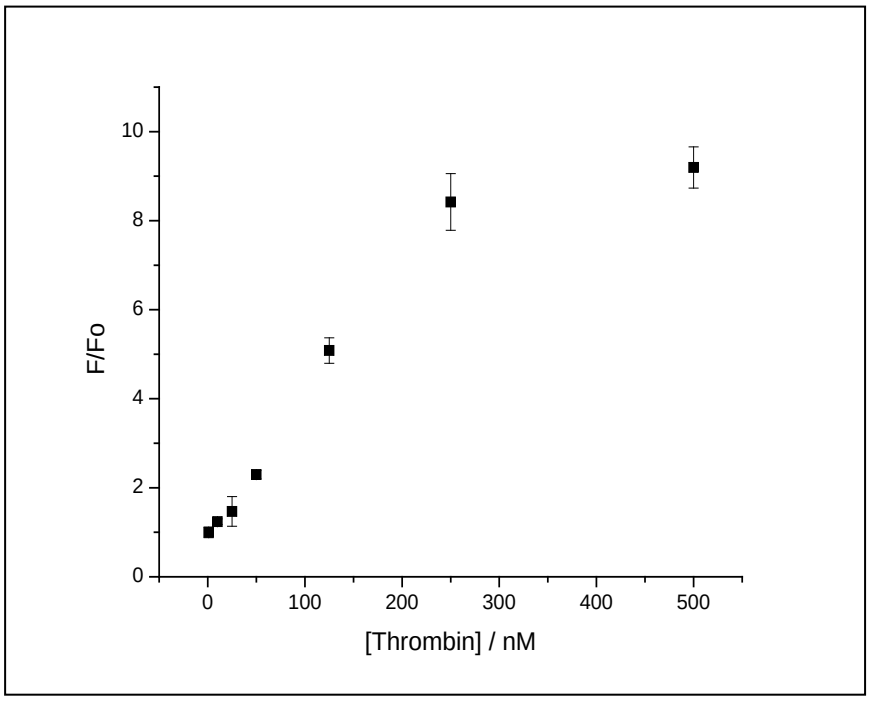
Bottom: Graph of Inflection points corresponding to maximum slope of the fluorescence curves from top graph

Experiment performed with same parameters of detection step and amplification step described in figures 8 and 9. Analysis done at 55°C for 5 minutes with fluorescence excitation of 490nm and emission 520nm

Figure 11. Thrombin target recognition schematic and performance







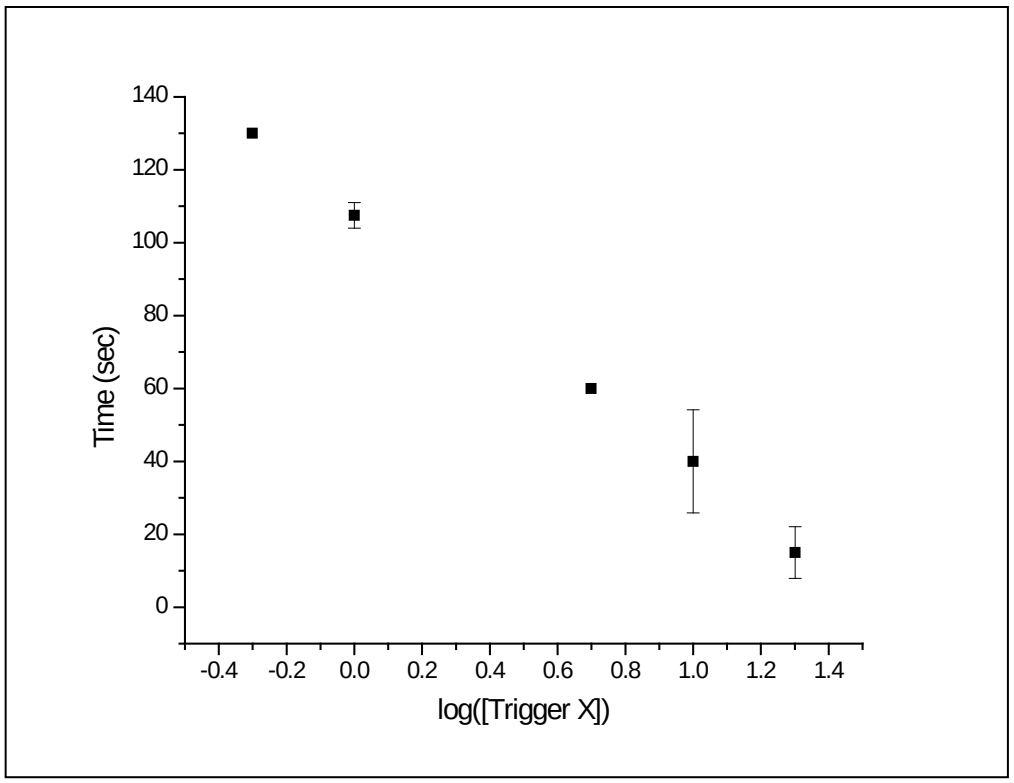
Top: Schematic of thrombin induced 'sandwich' formation showing binding of FAM-labeled Trigger X and subsequent release of Trigger X

Middle: Graph of increasing fluorescence intensity signal of FAM labeled Trigger X with increasing [Thrombin) = 0-500nM

Bottom: Graph of background to signal ratio of same Trigger X with increasing [Thrombin] = 0-500nM

Experiment performed on opaque neutravidin coated plates with 1.5 µM biotin labeled 15mer aptamer and 1.5nM FAM-labeled Trigger X with 29mer aptamer. Solutions incubated with 1mM biotin for 30 minutes and buffer containing varying [thrombin] for 30 minutes

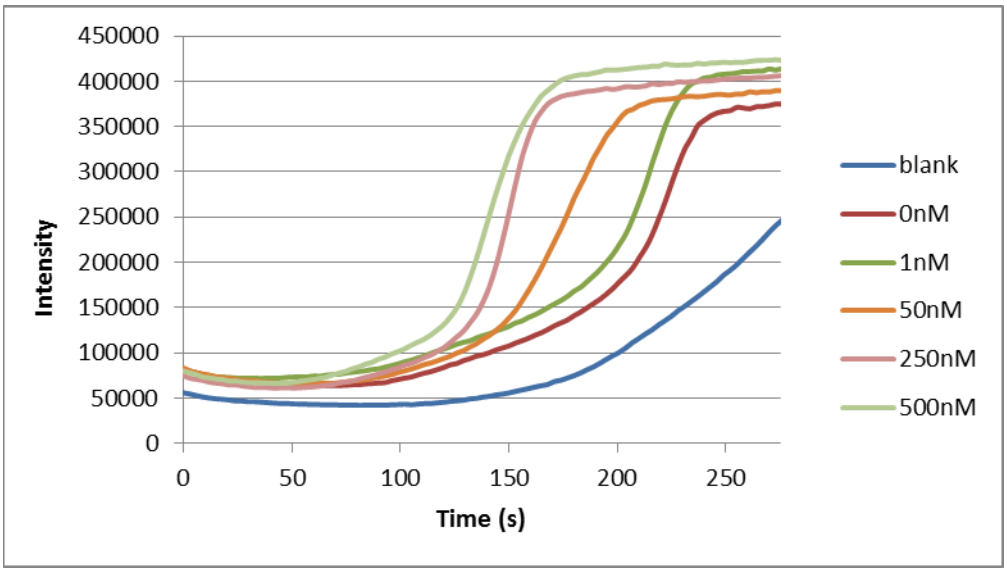
Figure 12. Thrombin Trigger X amplification via EXPAR

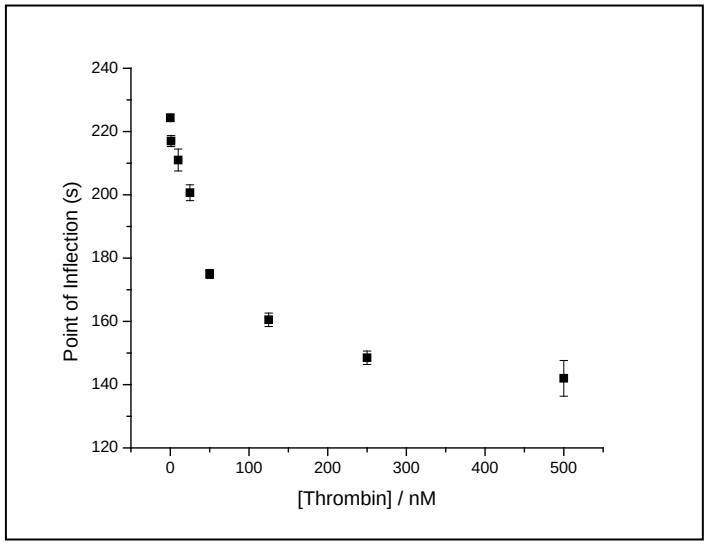


Graph of Inflection points corresponding to maximum slope of the fluorescence curves obtained from fluorescence intensity vs. time with varying [Trigger X]

Experiment performed by combing equal amounts of master mixes A and B and diluting Trigger X with millipore water to obtain its desired concentration prior to its insertion into master mix A. Samples analyzed for 5 minutes at 55°C with fluorescence excitation of 490nm and emission 520nm

Figure 13. Overall procedure (Thrombin): EXPAR of Trigger X released from sensor complex





Top: Graph of fluorescence intensity vs. time for Trigger X released after target induced binding of thrombin and Trigger X aptamer containing varying [thrombin], 0-500nM

Bottom: Graph of Inflection points corresponding to maximum slope of the fluorescence curves from top graph

Experiment performed with same parameters of detection step and amplification step described in figures 11 and 12. Analysis done at 55°C for 5 minutes with fluorescence excitation of 490nm and emission 520nm

CHAPTER 5

CONCLUSIONS

The system described here is a new method for the ultrasensitive detection of a target species using aptasensors based on exponential amplification reaction (EXPAR). It combines the highly selective and sensitive target recognition from aptamers with the rapid and facile DNA amplification of EXPAR and the total assay from target introduction to readout takes less than 30 minutes. With the general design of this system, the choice of aptamer is not limited and its activity can be maintained and optimized without disturbing the DNA amplification reaction that follows. It is proven that the aptamer maintains its activity after immobilization onto a solid surface, and that the design of the system enables optimizations of both target recognition by aptamer and DNA amplification by EXPAR. A key concept to this project is the careful yet simple design of the Trigger X which is incorporated into both systems. Trigger X is designed for utilization in the detection system and to provide a trigger for activation of the amplification system. This model of Trigger X allows for the systems to be coupled and optimized. As a proof of concept, a cocaine aptamer was chosen for target recognition of a small molecule. In the presence of cocaine, a structure switching process takes place which then releases an oligonucleotide complementary to the aptamer of choice (Trigger X). By using a FAM labeled Trigger X, we have shown that our detection system without coupling it to the performance enhancer has an LOD consistent with similar systems previously published (5μM in our case). [20] We also show that the isothermal amplification via EXPAR of our Trigger X which is designed so that it would be functional in both systems is achieved down to picomolar

concentrations. The coupling of the detection system with the amplification system is demonstrated with a LOD for cocaine of 800nM to 1μM, which is five times lower than the detection system alone without EXPAR. This work is the first to use this type of model of Trigger X to couple both systems. The cocaine detection system employs an aptamer structure switching model which depends on DNA hybridization. To exhibit the generalization of this method, changing to a target recognition system that utilizes a binding mechanism, similar to that in the thrombin binding aptamers, was also successfully coupled and provided a ten times enhancement to the LOD from 4nM to 400pM using performance enhancing amplification. This change was as simple as making a minor adjustment to the procedure to account for the change in the introduction of Trigger X to the amplification system, and slight adjustments to the Trigger X which did not compromise the original model design. This design of Trigger X showed that with as many as 38 non-active nucleotides, EXPAR of the trigger DNA is still maintained and reproducible.

For future efforts another minor modification that can be made to the system is the introduction of gold nanoparticles (AuNPs) for an ultrasensitive colorimetric detection method. This is accomplished through the incorporation of a second DNA template during the EXPAR scheme. [30] The second template translates the AmpDNA to another DNA strand that can serve as a bridging oligonucleotide for aggregating AuNPs and resulting in a color change from red to blue. The employment of this method into our system would be as easy as adding in the second template to the master mix prior to the addition of the Trigger X into the system. We anticipate that with these additions to our system, a general methodology for real-time, on-site and ultrasensitive detection of a wide range of targets is in the near future.

CHAPTER 6

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